Impact of genetic alterations on central nervous system progression of primary vitreoretinal lymphoma

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Abstract

Primary vitreoretinal lymphoma (PVRL) is a rare malignant lymphoma subtype with an unfavorable prognosis due to frequent central nervous system (CNS) progression. Thus, identifying factors associated with CNS progression is essential for improving the prognosis of PVRL patients. Accordingly, we conducted a comprehensive genetic analysis using archived vitreous humor samples of 36 PVRL patients diagnosed and treated at our institution and retrospectively examined the relationship between genetic alterations and CNS progression. Whole-exome sequencing (N=2) and amplicon sequencing using a custom panel of 107 lymphomagenesis-related genes (N=34) were performed to assess mutations and copy number alterations. The median number of pathogenic genetic alterations per case was 12 (range, 0-22). Pathogenic genetic alterations of *CDKN2A*, *MYD88*, *CDKN2B*, *PRDM1*, *PIM1*, *ETV6*, *CD79B*, and *IGLL5*, as well as aberrant somatic hypermutations, were frequently detected. The frequency of *ETV6* loss and *PRDM1* alteration (mutation and loss) was 23% and 49%, respectively. Multivariate analysis revealed *ETV6* loss (hazard ratio [HR]=3.26, 95% confidence interval [CI]: 1.08–9.85) and *PRDM1* alteration (HR=2.52, 95% CI: 1.03–6.16) as candidate risk factors associated with CNS progression of PVRL. Moreover, these two genetic factors defined slow-, intermediate-, and rapid-progression groups (0, 1, and 2 factors, respectively), and the median period to CNS progression differed significantly among them (52 *vs*. 33 *vs*. 20 months, respectively). Our findings suggest that genetic factors predict the CNS progression of PVRL effectively, and the genetics-based CNS progression model might lead to stratification of treatment.

Introduction

Primary vitreoretinal lymphoma (PVRL) is a malignant lymphoma subtype with lesions limited to the vitreous humor, retina, and optic nerve.[1] The pathological classification of PVRL is typically diffuse large B-cell lymphoma (DLBCL)[2] with *MYD88* L265P and/or *CD79B* mutations.[3,4]
Intravitreal chemotherapy, such as methotrexate (MTX), and local radiotherapy have been reported to achieve intraocular complete response and improve visual symptoms.[5-7] Additionally, systemic chemotherapy is often administered following local treatment in an effort to prevent subsequent central nervous system (CNS) progression. We previously reported a single-arm prospective study on newly diagnosed PVRL patients who received an intravitreal MTX injection followed by systemic high-dose MTX (HD-MTX). All patients achieved intraocular complete response, and the adverse events were generally tolerable.[8] However, the high rate of CNS progression indicated that these prophylactic strategies did not improve PVRL prognosis.[9,10] Therefore, the identification of factors associated with CNS progression is essential to improve the prognosis of PVRL patients.
In recent years, comprehensive genetic analyses using next-generation sequencing have revealed numerous genetic alterations in systemic DLBCL and provided solid evidence to newly classify DLBCL based on genetic alterations;[11-13] PVRL is classified into MCD/cluster 5 subtype with *MYD88* and *CD79B* mutations. Furthermore, genetic subtype-guided immunochemotherapy was reported to show better efficacy than conventional chemotherapy in

DLBCL.[14,15] Thus, this genetic approach is expected to be applied in clinical settings, such as exploring new target therapies and prognostic stratification.

We recently performed a retrospective analysis of PVRL patients diagnosed and treated at our hospital to identify the clinical factors associated with CNS progression, revealing bilateral disease and the detection of B-cell clonality confirmed via flow cytometry at diagnosis as risk factors.[16] Previously, we conducted direct sequencing and allele-specific polymerase chain reaction (PCR) to check the mutation of *CD79B* Y196 and *MYD88* L265P on the archived vitreous humor samples from 17 patients with PVRL and argued that *CD79B* Y196 potentially has a prognostic potential for patients with PVRL.[3] In the present study, we performed a comprehensive and massive genetic analysis of archived vitreous humor samples from 36 PVRL patients to identify genetic alterations strongly associated with CNS progression.

Methods

Patients

We enrolled 36 PVRL patients diagnosed from April 2012 to March 2022 who were treated at our hospital and had archived vitreous humor samples. Some of them have been included in previous studies,[3,8,16] and eight of 36 patients herein were identical to those in the previous study.[3] PVRL was defined as VRL localized to the eyes and was diagnosed as previously described.[8,9,16] Details of PVRL patients in this study are shown in Figure 1. The methods of FCM analysis, PCR analysis of *IGH* rearrangement, and cytokine measurement were previously described[3,8] and shown in the *Online Supplementary Appendix*. Treatment for the patients were also described in the *Online Supplementary Appendix*. This study was performed in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Tokyo Medical and Dental University (approval number: M2017-341). All patients provided written informed consent.

DNA extraction and next-generation sequencing

Genomic DNA was extracted from vitreous humor or from formalin-fixed paraffin-embedded (FFPE) brain tissue biopsies of PVRL patients using an EZ1 Virus Mini Kit v2.0 (QIAGEN, Hilden, Germany) or a QIAamp DNA FFPE Advanced Kit (QIAGEN), respectively. EZ1 Virus Mini Kit v2.0 usually extracts cell-free DNA; however, Zong *et al.* reported[17] that genomic DNA with enough quality and quantity was extracted from cells. Library preparation for amplicon-based targeted sequencing was performed as previously described[18] using a custom gene panel of 107 genes frequently mutated in lymphoma, particularly in PVRL (*Online Supplementary Table S1*). Briefly, the library was prepared using AmpliSeq Library Plus for Illumina (Illumina, San Diego, CA, USA). Further, synthesized libraries were sequenced in Miseq (Illumina) paired-end runs. The details of library synthesis method are described in the *Online Supplementary Appendix*.

Gene alteration analysis

We used a mutational analysis pipeline based on previously reported method.[18] The data handling step and used tools were described in the *Online Supplementary Appendix*. Variants considered pathogenic were identified according to previous reports (*Online Supplementary Table S1*). We also identified and counted aberrant somatic hypermutation (aSHM) to specify hypermutated cases, although their pathogenicity could not be determined. Copy number alterations (CNA) were calculated based on a previously

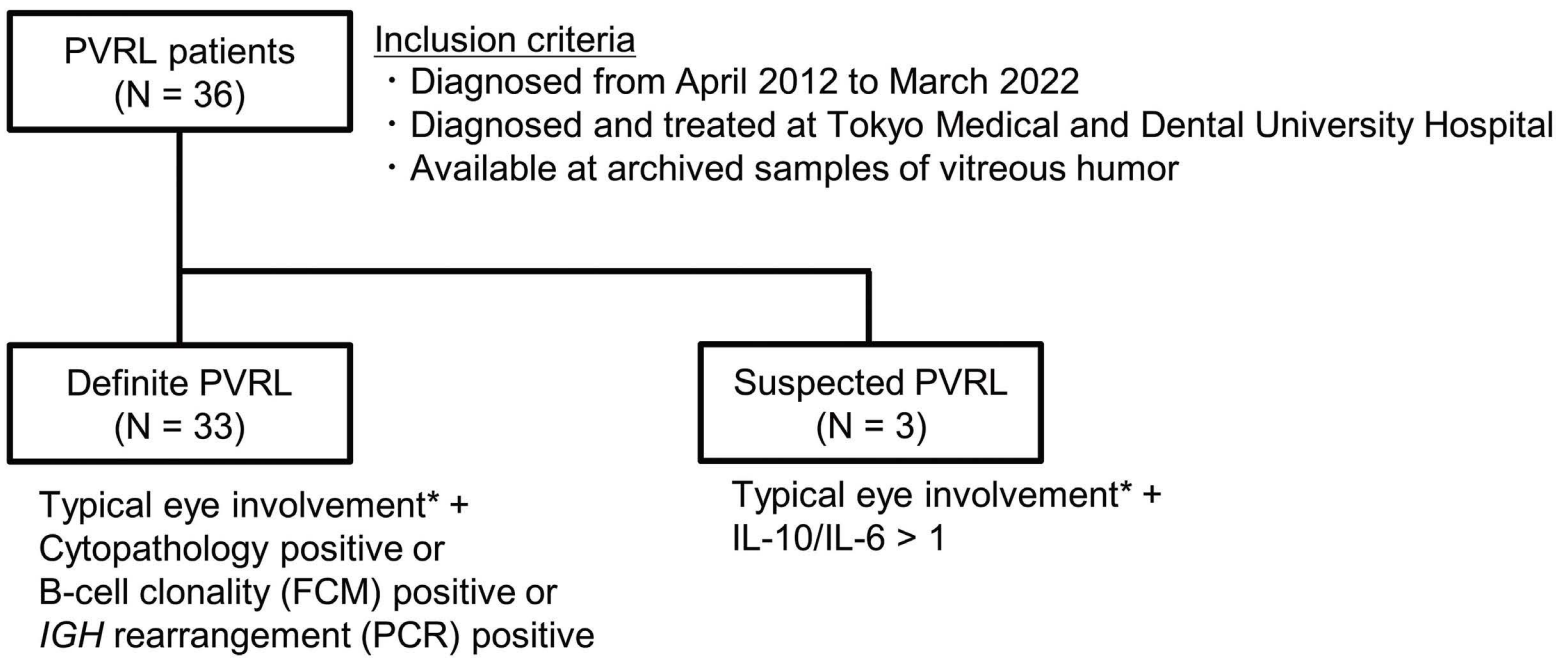


Figure 1. Classification of primary vitreoretinal lymphoma patients in this study. *Vitreous humor opacity and/or retinal or subretinal proliferative lesions. PVRL: primary vitreoretinal lymphoma; FCM: flow cytometry; PCR: polymerase chain reaction; IL: interleukin.

described method.[19] Detection methods of CNA were described in the *Online Supplementary Appendix* in detail.

Statistical analysis

Fisher exact test was used for categorical variable analysis and Mann-Whitney U test for continuous variable analysis. The cumulative incidence of CNS progression was calculated in the presence of death as a competing event, and the difference was tested using Gray test, although all PVRL patients who died had CNS progression before death. Factors used for the multivariate analysis were selected using the stepwise Akaike information criterion (AIC) method from the factors that revealed significant differences in the univariate analysis. Multivariate analysis was performed using Fine and Gray proportional hazard modeling. AIC was used as the selection criteria. All statistical analyses were performed using R 4.2.0 software (The R Foundation for Statistical Computing), and statistical significance was defined as $P<0.05$ based on a two-sided test.

Results

Patient characteristics

We evaluated 36 patients diagnosed with PVRL. The median follow-up period was 29 months (range, 2-119 months). Patient characteristics are summarized in Table 1. Ocular involvement was unilateral in 16 and bilateral in 20 patients. The median time from the onset of initial visual symptoms to diagnosis was 8 months (range, 1-29 months). Cytopathology, flow cytometry analysis, and *IGH* rearrangement were positive for PVRL in 42%, 74%, and 80% of patients, respectively. All 36 patients received intravitreal MTX injections following PVRL diagnosis, and 20 of 36 patients were treated with systemic HD-MTX thereafter. During the observation period, 19 patients developed CNS progression. Among the patients suspected with PVRL, one patient had CNS progression and ocular relapse, and one patient had ocular relapse.

Landscape of pathogenic genetic alterations in primary vitreoretinal lymphoma

Whole-exome sequencing (N=2) and amplicon sequencing using a custom panel containing 107 lymphomagenesis-related genes (N=34) were performed on vitreous humor samples to assess mutations and CNA. Average coverage depths of whole-exome sequencing were 105.8 and 143.5, and the mean coverage depth of amplicon sequencing was 621.8 (range, 74.8-1,098.0). One sample had low-quality DNA and could not be evaluated for CNA. The detected pathogenic gene mutations and CNA are presented in *Online Supplementary Tables S2* and *S3*, respectively.

At least one pathogenic genetic alteration was detected in 31 of 36 samples, and the median number of pathogenic genetic alterations per case was 12 (range, 0-22) (Figure 2A). The landscape of pathogenic genetic alteration is shown in Figure 2B. The top three altered genes were *CDKN2A* (25/36, 69%), *MYD88* (23/36, 64%), and *CDKN2B* (21/36, 58%). Of the 25 cases with altered *CDKN2A*, 23 showed copy number loss, two of which also showed mutation, and mutation only was observed in two cases. All *CDKN2B* alterations were copy number loss, and all *MYD88* alterations were mutations in p.Leu265. The other frequently altered genes were *PRDM1* (47%), *PIM1* (44%), *ETV6* (42%), *CD79B* (42%), and *IGLL5* (42%). In some cases, mutation and copy number loss were observed in the same genes. The cases with gene mutations showing >50% variant allele frequencies in the presence of copy number loss were identified. These alterations occurred in different alleles, indicating deletion of the normal allele.

Aberrant somatic hypermutation

Activation-induced deaminase mediates SHM and class-switch recombination by converting cytosine residues into uracil residues. aSHM arises from errors during SHM and occurs in genes other than *IGV* such as *PIM1* and *IGLL5*. aSHM is frequently detected in DLBCL, a subtype that accounts for most PVRL cases. However, the association between aSHM and DLBCL initiation has yet to be verified.[20,21] We picked up eight genes (*PIM1*, *OSBPL10*, *MPEG1*, *IGLL5*, *BTG1*, *BTG2*, *ETV6*, and *IRF4*), which were reportedly related to aSHM and examined the impact of aSHM in our study. Figure 3A

Table 1. Patient characteristics at primary vitreoretinal lymphoma diagnosis (N=36, unless otherwise indicated).

Characteristic	Value
Age in years, median (range)	71 (43-84)
Sex: male/female, N/N	14/22
Laterality: unilateral/bilateral, N/N	16/20
Initial visual symptoms, N (%)	
Blurred vision	20 (56)
Decreased vision	11 (31
Floaters	6 (17)
Photopsia	1 (3)
Sites involved, N (%)	
Vitreous body	34 (94)
Retina or subretinal site	18 (50)
Optic nerve	1 (3)
Time to diagnosis in months, median (range)	8 (1-29)
Cytopathology positive, class ≥IV, N (%)	15 (42)
B-cell clonality by FCM analysis, N/N (%)	23/31 (74)
Positive for *IGH* rearrangement by PCR, N (%)	28 (80)
Cytokine levels in the vitreous humor	
IL-10 pg/mL, median (range)	993.5 (10-130,125)
IL-10/IL-6 ratio >1, N (%)	33 (92)
Treatment received, N (%)	
Intravitreal MTX injection alone*	16 (44)
Intravitreal MTX injection + systemic HD-MTX	20 (56)

*Two patients received additional local radiation therapy. FCM: flow cytometry; HD-MTX: high-dose methotrexate; IL: interleukin; MTX: methotrexate; PCR: polymerase chain reaction.

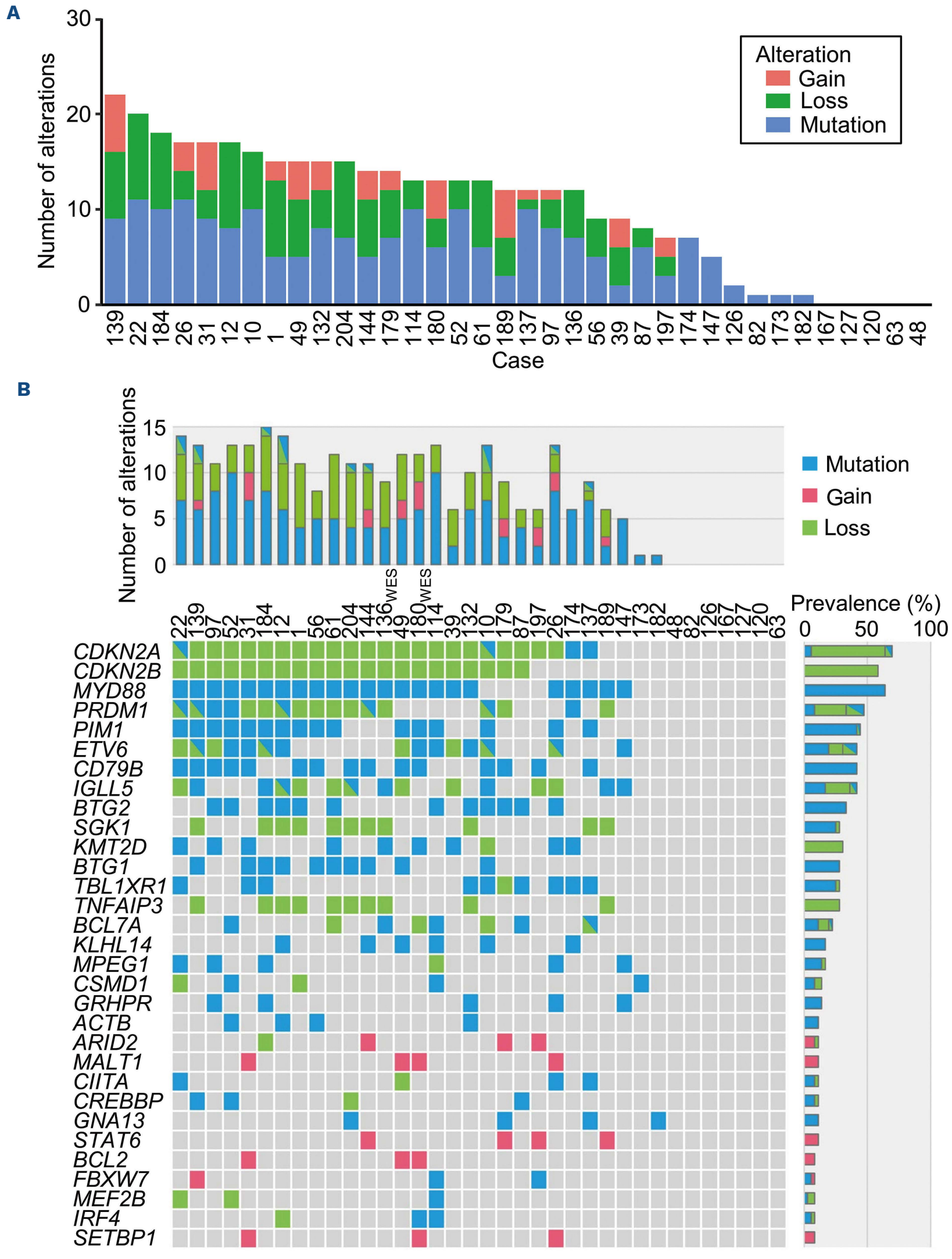


Figure 2. Pathogenic genetic alterations in primary vitreoretinal lymphoma. (A) Number of pathogenic genetic alterations per primary vitreoretinal lymphoma (PVRL) case. (B) Landscape of pathogenic genetic alterations in PVRL cases. Each column represents a case, and each row represents a recurrently altered gene. The bar graph on the right represents the frequency of pathogenic genetic alteration in each gene.

shows the number of mutations per gene per case. One or more mutations in *PIM1, OSBPL10*, *MPEG1*, *IGLL5*, *BTG1*, *BTG2*, *ETV6*, and *IRF4* were found in 24, 14, 12, 20, 10, 12, 12, and two of the 36 PVRL cases, respectively. Figure 3B shows the total number of mutations detected in these eight genes per case. The median number of mutations per case was ten (range, 0-35). There was no clear correlation between the number of pathogenic genetic alterations and the number of mutations detected in these eight genes related to aSHM per case.

Genetic risk factors associated with central nervous system progression

Figure 4A shows the cumulative incidence of CNS progression, and the 5-year cumulative incidence of CNS progression was 78.3%. We investigated possible genetic alterations associated with CNS progression. The univariate analysis identified *CD79B* mutation, *BTG1* mutation, *ETV6* loss, and *PRDM1* alteration (mutation and copy number loss) as candidate risk factors (Table 2). Factors used for the multivariate analysis were selected using the stepwise

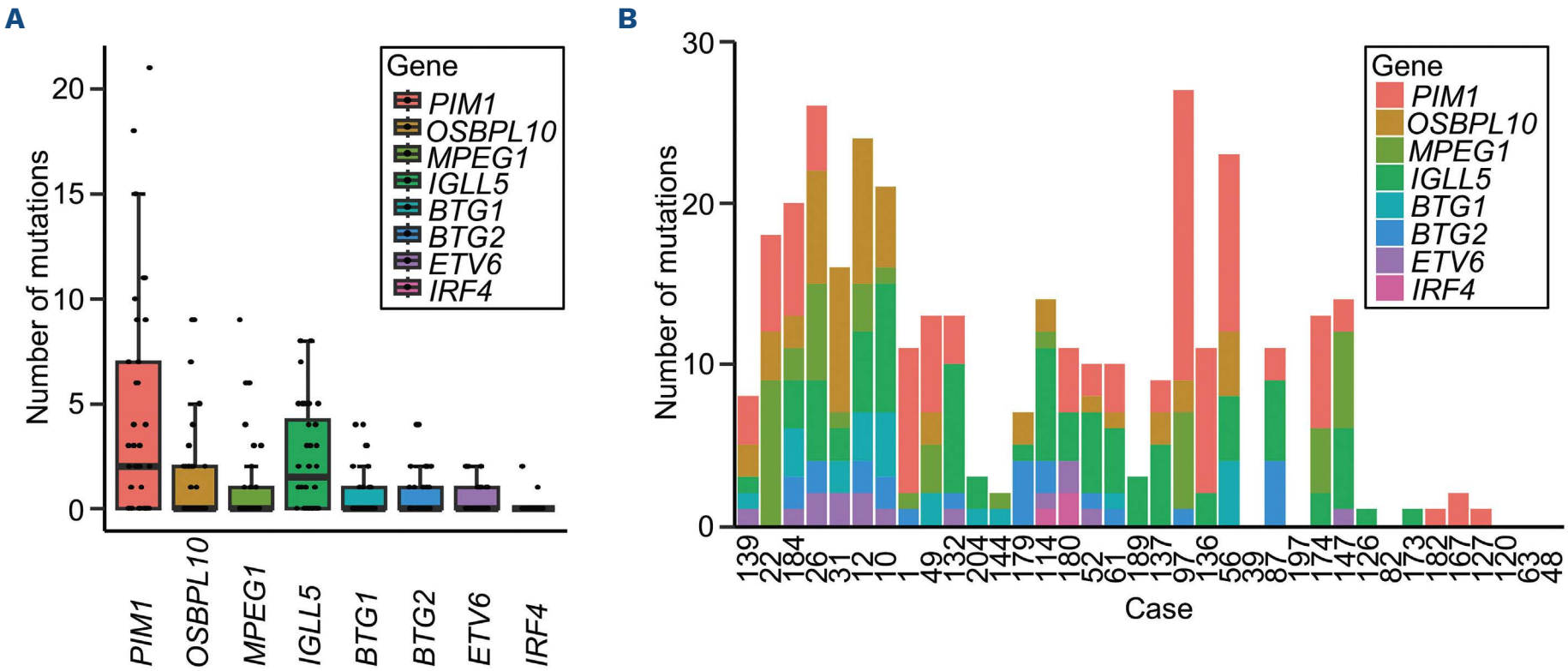


Figure 3. Aberrant somatic hypermutations of primary vitreoretinal lymphoma. (A) Number of mutations per gene per case. Each dot represents a case. (B) Total number of mutations detected in these 8 genes per case. The order of cases in the column is the same as in Figure 2A.

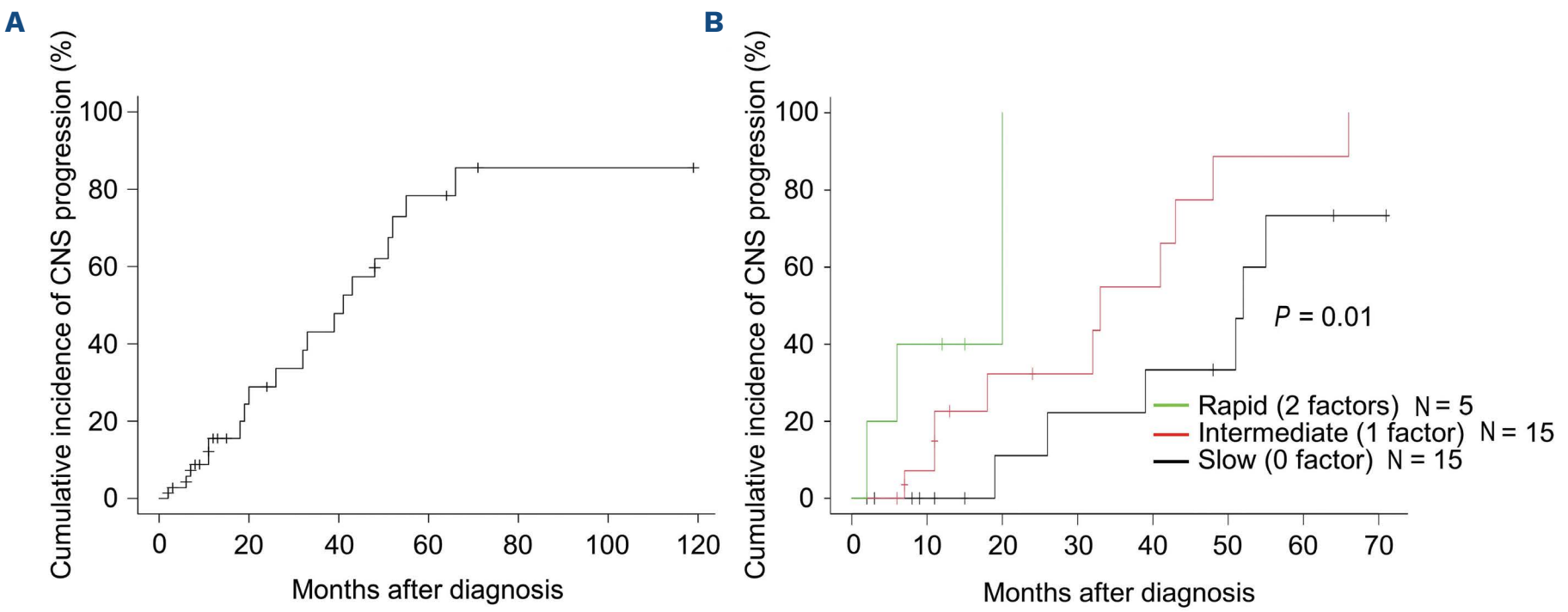


Figure 4. Genetic model of central nervous system progression in primary vitreoretinal lymphoma. (A) Cumulative incidence of central nervous system (CNS) progression in primary vitreoretinal lymphoma (PVRL). (B) Genetic model using *ETV6* loss and *PRDM1* alteration to define slow-, intermediate-, and rapid-progression groups (0, 1, and 2 factors, respectively). Cumulative incidence of CNS progression in the 3 groups is shown.

AIC method from these four factors, and *CD79B* mutation was excluded. *ETV6* loss and *PRDM1* alteration remained significant in the multivariate analysis (Table 2). The number of pathogenic genetic alterations and the number of mutations detected in eight genes related to aSHM were not associated with CNS progression. We also investigated the association between *ETV6* loss/*PRDM1* alteration and clinical findings of PVRL, but there was no significant correlation (*Online Supplementary Tables S4* and *S5*, respectively).

Genetic model of central nervous system progression in primary vitreoretinal lymphoma

ETV6 loss and *PRDM1* alteration were identified as risk factors for CNS progression in PVRL. We created a genetics-based CNS progression model using these two factors to define the slow-, intermediate-, and rapid-progression groups (0, 1, and 2 factors, respectively) (Figure 4B). The median period to CNS progression differed significantly among the three groups (52 *vs*. 33 *vs*. 20 months, respectively).

Genetic comparison between primary vitreous humor and brain samples

CNS progression occurred in 19 of 36 PVRL patients, four of whom underwent brain biopsy, and the tissue was processed using FFPE. The genetic-based group of the four patients was two in the intermediate-progression group and one in the slow- and rapid-progression groups. The period of CNS progression was 39 months (slow-progression group), 11 and 32 months (intermediate-progression group), and 20 months (rapid-progression group). We performed amplicon sequencing and analysis to compare pathogenic genetic alterations in the brain tissue samples with those in the vitreous humor samples taken at diagnosis. All four patients had at least one concordant alteration and had additional alterations that were found in the brain tissue samples but not in the vitreous humor samples (Figure 5). Details of detected pathogenic gene mutations and CNA in the brain tissue and vitreous humor samples are presented in *Online Supplementary Table S6*.

Table 2. Genetic risk factors for central nervous system progression.

Risk factor	Univariate analysis *P*	Multivariate analysis *P*	HR (95% CI)
CD79B mutation	0.03		
BTG1 mutation	0.01	0.08	2.31 (0.92-5.83)
ETV6 loss	0.04	0.04	3.26 (1.08-9.85)
PRDM1 alteration (mutation + loss)	0.04	0.04	2.52 (1.03-6.16)

P<0.05 were considered statistically significant. CI: confidence interval; HR: hazard ratio.

Discussion

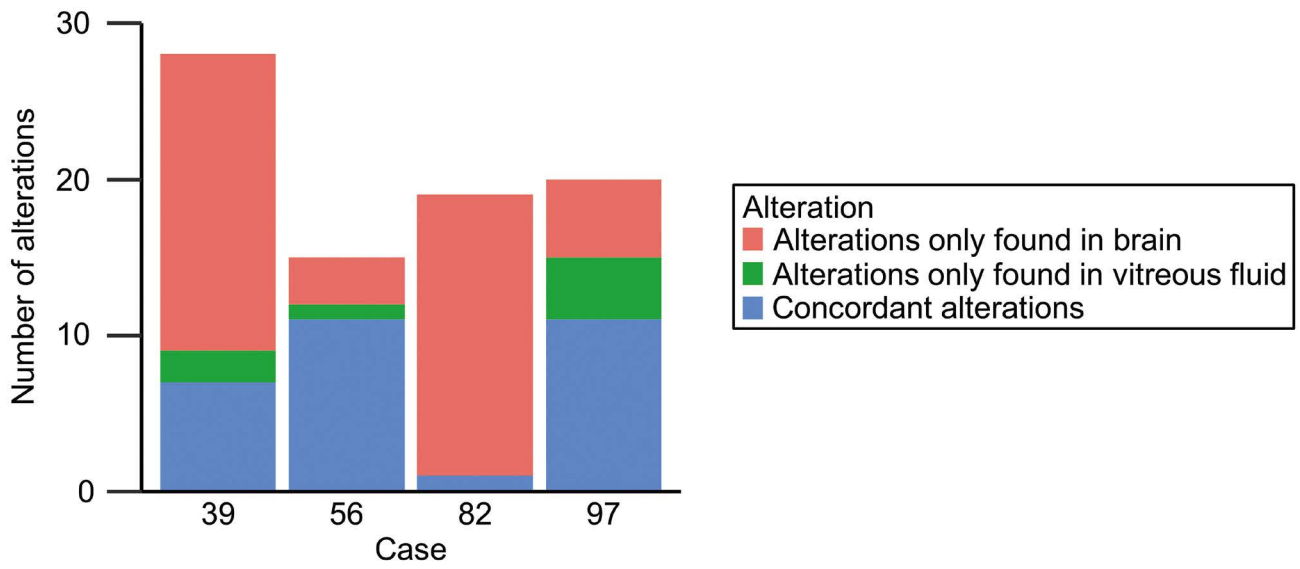
We conducted a comprehensive genetic analysis of 36 PVRL patients using vitreous humor samples taken at diagnosis and determined the genetic alterations related to CNS progression in PVRL.

Mutation and copy number analyses revealed that pathogenic genetic alterations of *CDKN2A*, *MYD88*, *CDKN2B*, *PRDM1*, *PIM1*, *ETV6*, *CD79B*, and *IGLL5* were common, as well as aSHM in *PIM1*, *OSBPL10*, *MPEG1*, *IGLL5*, *BTG1*, and *BTG2*. Due to the rarity of PVRL, comprehensive genetic analysis is challenging, and this is compounded by the low quantity and quality of DNA extracted from vitreous humor samples. However, a few groups have recently published exciting reports in this context using small amounts of DNA or cell-free DNA.[22-25] Because the results of our genetic analysis were highly consistent with the results of these comprehensive studies, we considered them suitable for the analysis of genetic alterations predictive of CNS progression in PVRL. Notably, there were 36 participants in our study, which is more than previous genetic analyses of PVRL.

We identified *ETV6* loss and *PRDM1* alteration (mutation and copy number loss) as candidate genetic alterations predicting CNS progression in PVRL. Our study is the first comprehensive genetic analysis to imply the association of genetic risk factors with CNS involvement. Thus, our findings stand out in terms of novelty.

ETV6 is a transcriptional repressor that plays a crucial role in hematopoiesis and is related to various types of hematological malignancies, including DLBCL.[11,26-28] *ETV6* loss, mutation, and fusion have been reported in primary central nervous system lymphoma (PCNSL).[29-31] This is consistent with our finding that *ETV6* loss is a factor related to CNS progression in PVRL, although the precise mechanism remains unclear. Notably, the level of ETV6 protein expression is negatively correlated with BIRC5 (survivin) expression and is associated with the antitumor effect of YM155, a BIRC5-specific inhibitor.[32] YM155 has shown clinical efficacy as a single agent or in combination with rituximab or bendamustine to treat relapsed/refractory DLBCL.[33,34] Future studies on the effectiveness of YM155 treatment for PVRL and its association with *ETV6* loss are anticipated.

PRDM1 is also a transcriptional repressor and a key molecule involved in plasma cell differentiation.[35] *PRDM1* mutation and loss are frequently detected in activated B-cell-like (ABC) DLBCL,[11] and conditional knockout of *PRDM1* in B cells results in constitutive NF-κB activation and the development of lymphoproliferative disorders resembling ABC-DLBCL *in vivo*.[36] Genetic alteration of *PRDM1* frequently occurs in PCNSL.[29] Although the precise mechanism of CNS progression remains undefined, considering that *PRDM1* alterations are infrequent in systemic extranodal DLBCL,[37,38] there may be a CNS-specific genetic pathogenesis. Bruton tyrosine kinase (BTK) inhibitors interfere with B-cell receptor and NF-



	39	56	82	97
Genetics-based CNS progression group	Intermediate	Intermediate	Slow	Rapid
Period to CNS progression	32 months	11 months	39 months	20 months

Figure 5. Genetic alterations at initial onset and after central nervous system progression. Number of pathogenic genetic alterations in vitreous humor samples taken at disease onset and brain tissue biopsy samples taken after central nervous system (CNS) progression per case. Concordant alterations (found in both vitreous humor and brain tissue) and discordant alterations (found in either vitreous humor or brain tissue) are indicated.

κB signaling by inhibiting BTK, and ibrutinib (a BTK inhibitor) has shown encouraging clinical activity against lymphomas involving the CNS and intraocular sites.[39] Thus, it would be interesting to investigate whether the presence or absence of genetic alterations in *PRDM1* affects BTK inhibitor efficacy. Moreover, Pascual *et al*. reported that constitutive NF-κB activation and impaired differentiation resulting from Blimp1 (a PRDM1 homolog) inactivation downregulated p53 signaling and triggered immune escape in ABC-DLBCL and that simultaneous PD-1 blockade improved the efficacy of anti-CD20 immunotherapy in an ABC-DLBCL-like mouse model.[40] In parallel, since the efficacy of PD-1 blockade for CNS lymphoma has been reported,[41,42] immune checkpoint modulation for PVRL patients with *PRDM1* alteration may be an intriguing therapeutic approach.

The genetics-based CNS progression model that we proposed in this study used two genetic alterations, namely, *ETV6* loss and *PRDM1* alterations, to successfully define three statistically significant groups for CNS progression in PVRL patients. To break through this intractable lymphoma, therapeutic strategies should be adapted using conventional HD-MTX-based chemotherapy regimens in potential combination with novel agents, such as BTK inhibitors, to the CNS progression risk of each patient. Our genetics-based CNS progression model might help this stratified treatment. Since a comparison between the genetic alterations in PVRL at disease onset and after CNS progression had never been reported, we performed a longitudinal comparison of pathogenic genetic alterations identified using amplicon sequencing of FFPE brain tissue samples from four PVRL patients with CNS progression and their vitreous humor samples at diagnosis. All four patients with PVRL had at least one concordant alteration. Balikov *et al.* conducted target sequencing of matched brain and vitreous samples in two PCNSL patients with VRL and showed shared genetic alterations, suggesting the same origin.[43] Similarly, our results described that brain lesions were of the same origin as the vitreous lesions at diagnosis. Moreover, all four patients had additional pathogenic genetic alterations that were absent at disease onset. Thus, future analysis with a larger number of PVRL patients may facilitate the identification of additional genetic alterations associated with CNS lesion development.

This study has some limitations. First, as a single-institute, retrospective analysis, selection bias cannot be ignored. Second, although we considered the number of lymphomagenesis-related genes (N=107) examined in the amplicon sequences sufficient to cover most genetic alterations in the context of PVRL, it is possible that other (untested) genetic alterations are involved in CNS progression. Third, although our study enrolled 36 PVRL patients, which is the largest in number to date for a comprehensive genetic analysis of this rare disease, the sample size was small. During the analysis of rare diseases, the small sample size might reduce the power of detection.[44] Therefore, we did not use multiplicity correction methods for the results of regression tests because they would have further reduced the detection power. Studies with large sample

sizes using multiple comparison correction methods in multivariate analysis will enable a detailed investigation of the biological characteristics of PVRL. Finally, we could not validate our results in another cohort. We seek to test the validity of this genetics-based CNS progression model in a prospective and large cohort through international collaborations in the future.

To summarize, our comprehensive genetic analysis identified *ETV6* loss and *PRDM1* alterations as candidate genetic risk factors related to CNS progression in PVRL. Subsequently, we created a new model for CNS progression using these two genetic risk factors. A prospective and large study is necessary to validate this model. With proven validity, interventions with new drugs targeting these genetic alterations in possible combination with other available therapeutic options based on this model may improve the outcome of PVRL.

Disclosures

No conflicts of interest to disclose.

Contributions

KYo and DS designed the study. KYo, YM, and HT collected the clinical data. HT and KYa collected the clinical samples. KYo, DS, TT, CH, and TN carried out the research and analyzed the data. KYo, DS, TT, and TN wrote the manuscript and designed the figures and tables. HT, YH, TM, and TN supervised the project. All authors approved the final manuscript.

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Data sharing statement

Requests for original data should be addressed to the corresponding author.

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